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Original Paper

Novel Insights in the Regulation of Phosphatidylserine Exposure in Human Red Blood Cells

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Key Words
Red blood cells • Ca^{2+} content • Phosphatidylserine exposure • Protein kinase C • Flow cytometry • Fluorescence imaging

Abstract
Background/Aims: In previous publications we were able to demonstrate the exposure of phosphatidylserine (PS) in the outer membrane leaflet after activation of red blood cells (RBCs) by lysophosphatidic acid (LPA), phorbol-12 myristate-13acetate (PMA), or 4-bromo-A23187 (A23187). It has been concluded that three different mechanisms are responsible for the PS exposure in human RBCs: (i) Ca^{2+}-stimulated scramblase activation (and flippase inhibition) by A23187, LPA, and PMA; (ii) PKCα activation by LPA and PMA; and (iii) enhanced lipid flip flop caused by LPA. Further studies aimed to elucidate interconnections between the increased Ca^{2+} content, scramblase- and PKCα-activation. In addition, the role of the Ca^{2+}-activated K^+ channel (Gardos channel) activity in the process of PS exposure needs to be investigated. ***Methods:*** The intracellular Ca^{2+} content and the PS exposure of RBCs have been investigated after treatment with LPA (2.5 µM), PMA (6 µM), or A23187 (2 µM). Fluo-4 and annexin V-FITC has been used to detect intracellular Ca^{2+} content and PS exposure, respectively. Both parameters (Ca^{2+} content, PS exposure) were studied using flow cytometry. Inhibitors of the scramblase, the PKCα, and the Gardos channel have been applied. ***Results:*** The percentage of RBCs showing PS exposure after activation with LPA, PMA, or A23187 is significantly reduced after inhibition of the scramblase using the specific inhibitor R5421 as well as after the inhibition of the PKCα using chelerythrine chloride or calphostin C. The inhibitory effect is more pronounced when the scramblase and the PKCα are inhibited simultaneously. Additionally, the inhibition of the Gardos channel using charybdotoxin resulted in a significant reduction of the percentage of RBCs showing PS exposure under all conditions measured. Similar results were obtained when

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the Gardos channel activity was suppressed by increased extracellular K^+ content. ***Conclusion:*** PS exposure is mediated by the Ca^{2+}-dependent scramblase but also by PKCα activated by LPA and PMA in a Ca^{2+}-dependent and a Ca^{2+}-independent manner. Furthermore, we hypothesize that a hyperpolarisation of RBCs caused by the opening of the Gardos channel is essential for the scramblase activity as well as for a fraction of the LPA-induced Ca^{2+} entry.

Introduction

When the endothelium of blood vessels is damaged, platelets become activated and transport phosphatidylserine (PS) to their external membrane surface [1]. The exposed PS provides a catalytic surface for the formation of active enzyme-substrate complexes of the coagulation cascade, especially for the tenase and prothrombinase complexes [2]. Under these circumstances exposed PS provides a pro-coagulant surface and is, in general, needed as a response to injury. So it is logical that the mechanism of PS exposure has to occur with a relative high transport rate of the lipids. Platelets treated with a Ca^{2+} ionophore show a scrambling rate of 87×10^{-3} per second [2]. Human red blood cells (RBCs) also show the mechanism of PS exposure after increased intracellular Ca^{2+} content [3-5] and are able to adhere to endothelial cells under pathophysiological conditions [6-9]. In addition, exposure of PS at the external surface of the membrane of RBCs is a typical sign of eryptosis (a term introduced by Lang et al. [10]), defining the suicidal death of RBCs. Exposed PS is sought to serve as a signaling component for macrophages to eliminate old or damaged RBCs from the circulation [11-14]. A large variety of physiological parameters as well as substances have been described to induce eryptosis (e.g., [15 – 18]). Since eryptotic RBCs can adhere to the vascular wall, which may lead to disturbance of the microcirculation [19], the elimination of these cells is a very important mechanism. Beyond that, it was also shown that RBCs with an increased Ca^{2+} content adhere towards each other under *in vitro* conditions [20]. However, compared to platelets, RBCs have a lower scrambling rate (0.45×10^{-3} per second) [2].

The outward-directed transport of PS is realized by a protein stimulated by an increased intracellular Ca^{2+} content termed 'scramblase' [21-23]. The molecular identity of this protein has been determined only recently as a member of the TMEM16 or anoctamin family of proteins and the crystal structure was published [24].

Ca^{2+} uptake of RBCs through Ca^{2+}-permeable channels [25-28] does not only activate the scramblase, it also leads to an activation of the Gardos channel [29, 30] also known as hSK4, KCNN4 or $K_{Ca}3.1$. The result is an efflux of KCl and osmotically obliged H_2O, which causes shrinkage of the cells [30, 31].

Another consequence of increased intracellular Ca^{2+} content is the translocation of the protein kinase Cα (PKCα) to the plasma membrane as the initial step of their activation [25, 32]. Since mature human RBCs lack nuclei and organelles, cellular responses have to be modulated by post-translational modifications. Therefore, phosphorylation mediated by the PKCα is of great importance for intracellular signal transduction [33, 34]. In addition, it has been discussed that an activation of the PKCα results in an enhanced uptake of Ca^{2+} into the cells, i.e. acting as a positive feedback [35]. It has also been speculated that an activation of the PKCα induces PS exposure via a Ca^{2+}-independent mechanism [35].

The current paper is a continuation of our previous work on the mechanism of PS exposure in stimulated RBCs [3, 5, 25, 36-38]. Several substances can be applied to modulate the Ca^{2+} homeostasis and phospholipid distribution. In addition to a variety of transport inhibitors, we apply R5421, an inhibitor of the scramblase, which commercial availability we initiated.

From the results obtained it has been concluded that three different mechanisms are responsible for the PS exposure in human RBCs: (i) Ca^{2+}-stimulated scramblase activation (and flippase inhibition) [3], whereas the Ca^{2+} influx is mediated by two distinct pathways,

an ω-agatoxin-TK-sensitive pathway ($Ca_V2.1$-like channel) and a PKCα-dependent signalling [25]; (ii) a direct action of the PKCα and possibly PKCζ on the PS exposure by phosphorylation of an unknown target protein [3]; and (iii) enhanced lipid flip flop caused by LPA [3].

Therefore, the aim of this paper was to further characterize the relation between the increased Ca^{2+} content, PKCα activation, and PS exposure of RBCs. Furthermore, we considered the role of the Gardos channel, cell volume changes and/or changes of the K^+ concentration in the process of PS exposure.

Material and Methods

Blood and solution

Human venous blood from healthy human volunteers was obtained from the Institute of Clinical Haematology and Transfusion Medicine, Saarland University Hospital, Homburg, or from the Institute of Sports and Preventive Medicine, Saarland University, Saarbruecken. EDTA or heparin was used as anticoagulants. Freshly drawn blood samples were stored at 4°C and used within one day as recently recommended [39]. Blood was centrifuged (2,000 g, 5 min) at room temperature and the plasma and buffy coat was removed by aspiration. Subsequently, RBCs were washed 3 times in HEPES-buffered physiological solution (HPS) containing (mM): 145 NaCl, 7.5 KCl, 10 glucose, 10 HEPES, pH 7.4 under the same conditions. Finally, RBCs were re-suspended in HPS and stored at 4°C until the beginning of the experiment. The experiment was started immediately after resuspension of the cells.

RBC labelling

The procedure to prepare RBCs for measurements of intracellular Ca^{2+} content as well as PS exposure is based on the protocols of Nguyen et al. [3], Wesseling et al. [37, 38], and Kucherenko and Bernhardt [40].

Measurement of intracellular Ca^{2+} content: RBCs were loaded with 1 µM fluo-4 AM from a 1 mM stock solution in dimethyl sulfoxide (DMSO) in 2 ml HPS as described before [3, 37, 38]. The extracellular Ca^{2+} concentration was 2 mM, i.e. $CaCl_2$ was added to the HPS. Cells were incubated at a haematocrit of about 0.1 % in the dark for 30 min at 37°C with continuous shaking. For comparison, some experiments were carried out with lower extracellular Ca^{2+} concentrations as well as lower incubation times. Then the cells were washed again (16,000 g, 10 s) with an ice-cold HPS, re-suspended and used for measurements, i.e. for control measurements or for activation by different substances (A23187, LPA, PMA). It has to be mentioned that 2 different LPA batches from the same company (see Reagents) were used for the experiments. However, in a recent publication we showed that the percentage of RBCs showing PS exposure strongly depends on the LPA batch, even if obtained from one company [38].

Measurement of PS exposure: PS exposure was detected using annexin V-FITC at a concentration of 4.5 µM. The cells were prepared as for measurement of the Ca^{2+} content. The RBCs were incubated with different substances (A23187, LPA, PMA) between 1 min and 30 min at 37°C. Then the cells were washed again (16,000 g, 10 s) with an ice-cold HPS and re-suspended. Finally annexin V-FITC was added and the cells were incubated in HPS with the addition of 2 mM Ca^{2+} at a haematocrit of 0.1 % and room temperature for 10 min in the dark. The measurements were performed at room temperature.

Treatment of RBCs with different substances / under different experimental conditions

Cells in HPS containing additionally 2 mM $CaCl_2$ (haematocrit 0.1 %) were activated with A23187 or PMA for 30 min and with LPA for 1 min in Eppendorf tubes under continuous shaking at 37°C. This means that for RBC activation the last centrifugation was done in the presence of 2 mM $CaCl_2$. When chelerythrine chloride was used, the cells were pre-incubated for 20 min under the same conditions [41]. In case of pre-incubation with calphostin C, charybdotoxin, or R5421 the incubation time was 30 min [42, 43]. As stated above, some experiments were done in the presence of lower Ca^{2+} concentration for comparison. In addition, the activation time with A23187 was reduced to 1 min.

To avoid KCl efflux and cell shrinkage, i.e. to block the Gardos channel, RBCs were transferred into a high K^+ HEPES-buffered solution containing (mM): 150 KCl, 2.5 NaCl, 10 glucose, 10 HEPES, pH 7.4. Again, 2 mM $CaCl_2$ was added to the solutions before activating the RBCs with different substances. To change the volume of the RBCs, they were transferred into a sucrose-containing HPS containing (mM): 145 NaCl,

7.5 KCl, 2 $CaCl_2$, 10 glucose, 30 sucrose, 10 HEPES, pH 7.4 (for shrinkage) or into HPS with reduced NaCl concentration containing (mM): 130 NaCl, 7.5 KCl, 2 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4 (for swelling).

Flow cytometry and fluorescence microscopy

To analyse the RBCs we used the flow cytometer 'FACSCalibur' and the software Cell Quest Pro (Becton Dickinson Biosciences, Franklin Lakes, USA) as described before [3, 37, 38]. The fluo-4 and annexin V-FITC fluorescence signals were measured in the FL-1 channel, with an excitation wavelength of 488 nm and an emission wavelength of 520/15 nm. Forward scatter (FSC) was analysed to determine cell volume changes. For each experiment 30,000 cells were measured.

Fluorescence microscopy was carried out with the inverted fluorescence microscope Eclipse TE2000-E (Nikon, Tokyo, Japan) and the imaging software VisiView (Visitron Systems, Puchheim, Germany) as described before [3, 37, 38]. Images were taken with the camera CCD97 (Photometrics, Tucson, USA) using a 100×1.4 (NA) oil immersion lens with infinity corrected optics. Diluted RBC samples (haematocrit 0.1 %) were placed on a cover slip in the dark at room temperature. From each RBC sample 5 images from different positions of the cover slip randomly chosen were taken.

Reagents

Ca^{2+} ionophore A23187, lysophosphatidic acid (LPA), phorbol 12-myristate 13-acetate (PMA), chelerythrine chloride, calphostin C, and charybdotoxin were purchased from Sigma-Aldrich (Munich, Germany). All substances (except charybdotoxin, which was dissolved at 20 µM in HPS) were dissolved at 1 mM in DMSO and stored at -20°C. For each experiment a new aliquot was used. R5421 was obtained from Endotherm (Saarbruecken, Germany) where it has been synthesized according to the structure published by Dekkers et al. [23] (see Fig. 3 therein), dissolved at 100 mM in DMSO, and stored at room temperature. Fluo-4 AM and annexin V-FITC was obtained from Molecular Probes (Eugene, USA).

Statistics

Data are presented as mean values +/- S.D. of at least 3 independent experiments. The significance of differences was tested by ANOVA. Statistical significance of the data was defined as follows: (***): $p \leq 0.001$, (**): $p \leq 0.01$, (*): $p \leq 0.05$, not significant: $p > 0.05$.

Results

We have shown before that stimulation of RBCs with LPA, an increase of intracellular Ca^{2+}, and an activation of PKCα increased the percentage of PS exposing cells [3, 37]. However, the detailed interaction between these Ca^{2+}-dependent and Ca^{2+}-independent processes and the contribution of the involved players like the scramblase or the putative participation of the Gardos channel needed further investigations. Towards these investigations we used inhibitors of the scramblase, the PKCα and the Gardos channel for measurements of the intracellular Ca^{2+} content and PS exposure.

Inhibition of the scramblase

A pre-incubation with 100 µM of the scramblase inhibitor R5421 [23] leads to different results when the RBCs are activated with A23187, LPA, or PMA (Fig. 1A, B). For A23187, a pre-incubation with R5421 does not affect the percentage of cells with elevated intracellular Ca^{2+} content. In case of LPA, the number of cells with elevated intracellular Ca^{2+} is slightly increased after incubation with R5421 compared to control. For PMA the opposite effect can be seen, i.e. the percentage of RBCs with increased intracellular Ca^{2+} content is significantly reduced after incubation with R5421. The control value for the RBC number with elevated Ca^{2+} content in the absence of any activating substance was 0.80 ± 0.09 % in the absence of R5421 and DMSO, 1.19 ± 0.17 % in the absence of R5421 and presence of DMSO, and 1.12 ± 0.20 % in presence of R5421 and DMSO (n = 5, no pair of the 3 values shows a significant difference).

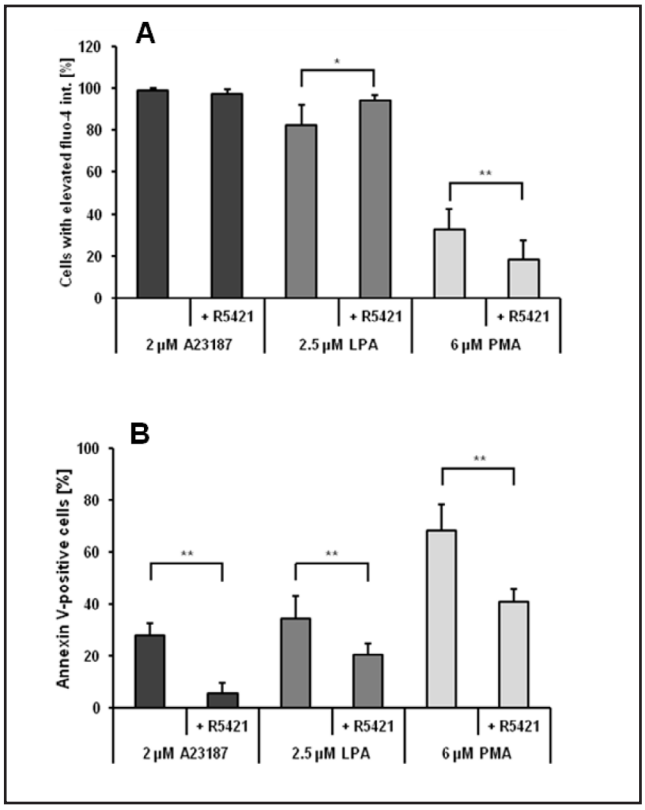

Fig. 1. Percentage of RBCs (A) responding with increased intracellular Ca^{2+} content (elevated fluo-4 intensity) and (B) responding with increased PS exposure (annexin V-positive cells) after activation with A23187 (2 µM) for 30 min, LPA (2.5 µM) for 1 min, or PMA (6 µM) for 30 min in the absence or presence of the scramblase inhibitor R5421 (100 µM) using flow cytometry. Mean values of at least 5 different blood samples (5 x 30.000 cells), error bars = S.D. (only the upper error bars are shown for convenience). Significant differences, ANOVA ($0.01 < p \leq 0.05$ (*); $0.001 < p \leq 0.01$ (**)) are shown in the Figure. Please note that for the experiments presented in Figs. 1, 4, and 6 another LPA batch compared to experiments presented in Fig. 3 was used (this explains slight differences in the LPA control values, see also Material and Methods).

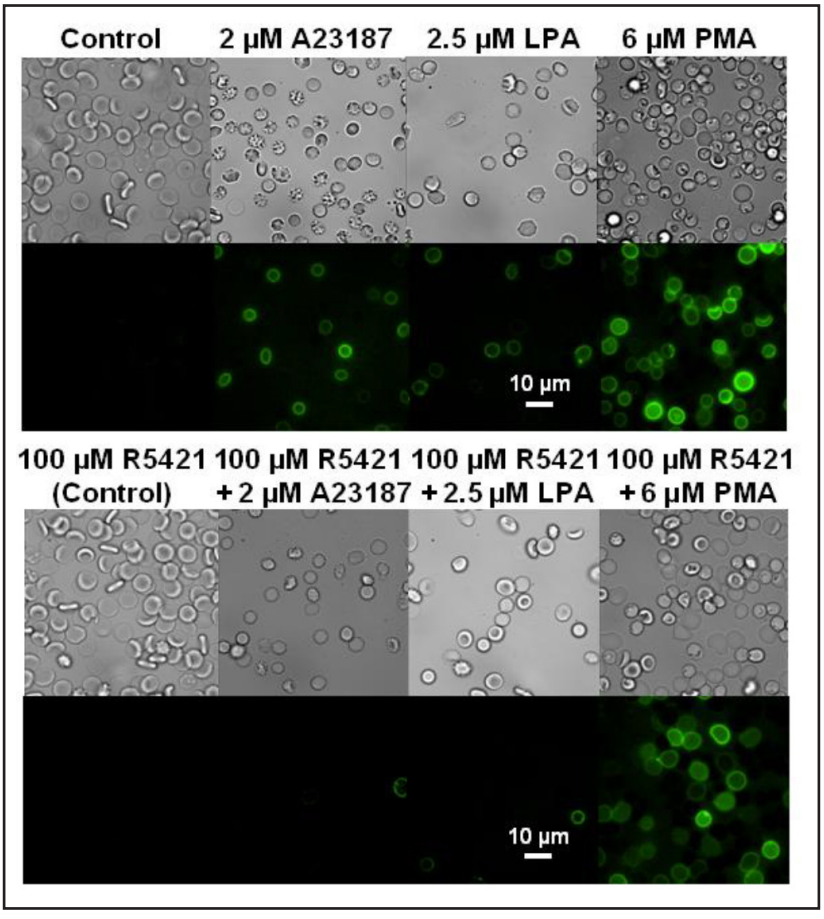

Fig. 2. Fluorescence microscopy images of RBCs after activation with A23187 (2 µM) for 30 min, LPA (2.5 µM) for 1 min, and PMA (6 µM) for 30 min as well as control (absence of any activating substance) in the absence or presence of the scramblase inhibitor R5421 (100 µM). R5421 has been added to the RBCs before activation. Upper rows – transmitted light, lower rows – fluorescence images to detect PS exposure using annexin V-FITC. RBCs in HPS with additional $CaCl_2$ (2 mM). Representative images out of 4 independent experiments.

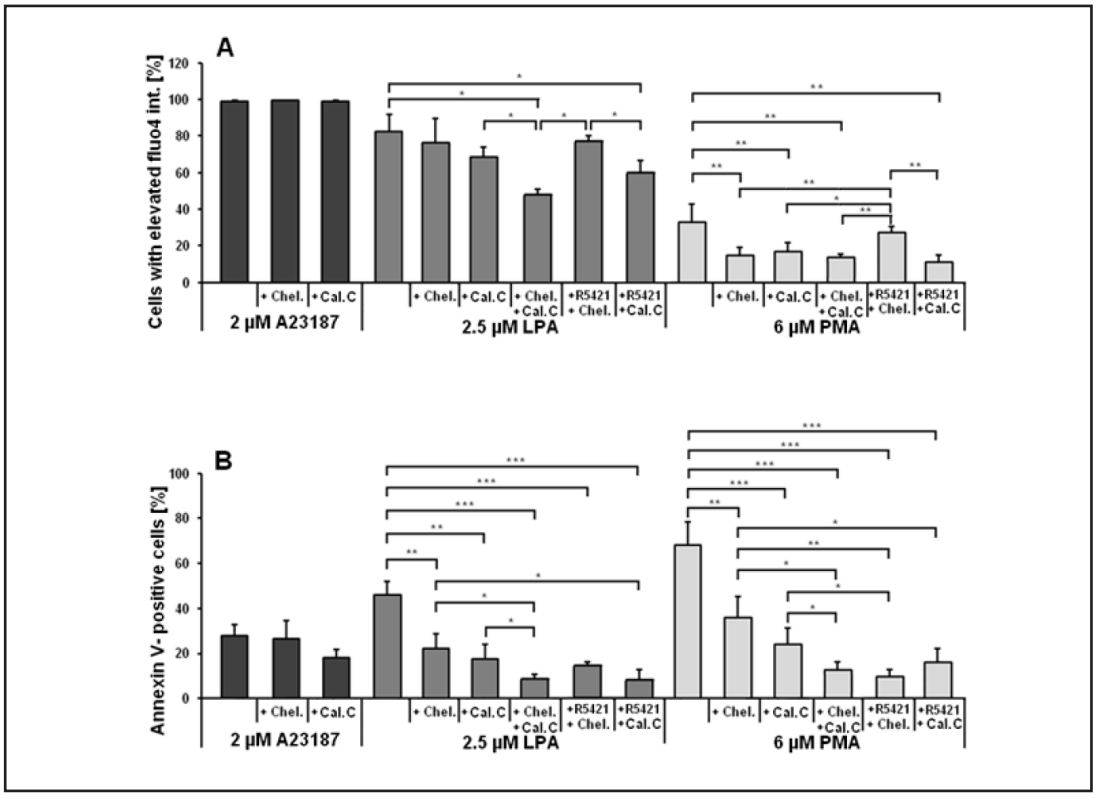

Fig. 3. Percentage of RBCs (A) responding with increased intracellular Ca^{2+} content (elevated fluo-4 intensity) and (B) responding with increased PS exposure (annexin V-positive cells) after activation with A23187 (2 µM) for 30 min, LPA (2.5 µM) for 1 min, or PMA (6 µM) for 30 min in the presence of the PKCα inhibitors chelerythrine chloride (Chel., 10 µM), calphostin C (Cal. C, 1 µM), both inhibitors together (Chel., 10 µM + Cal. C, 1 µM), chelerythrine chloride (Chel., 10 µM) plus the scramblase inhibitor R5421 (100 µM), or calphostin C (Cal. C, 1 µM) plus R5421 (100 µM), compared to control (absence of inhibitors) using flow cytometry. Two inhibitors have been applied together only in case of LPA or PMA stimulation. Mean values of at least 3 different blood samples (3 x 30.000 cells), error bars = S.D. (only upper error bars are shown for convenience). Significant differences, ANOVA ($0.01 < p \leq 0.05$ (*); $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***)) are shown in the Figure. Please note that for the experiments presented in Figs. 1, 4, and 6 another LPA batch compared to experiments presented in Fig. 3 was used (this explains slight differences in the LPA control values, see also Material and Methods).

We like to stress that A23187 served as positive control. We are aware that 2 mM extracellular Ca^{2+} is a substantial concentration leading to a much higher intracellular Ca^{2+} content compared with LPA- or PMA-stimulation. Therefore, we carried out experiments with A23187 activation in the presence of 0.1 mM, 0.5 mM, 1 mM, and 2 mM $CaCl_2$ in the extracellular solution and measured the number of cells with increased intracellular Ca^{2+} content after A23187 activation between 1 and 30 min. At all A23187 concentrations and at all activation times nearly 100 % of the cells reacted with an increased Ca^{2+} content.

The situation for PS exposure is different. As shown in Fig. 1B, pre-incubation with R5421 leads to a significant reduction of the percentage of RBCs showing PS exposure in all three cases of activation (A23187, LPA, PMA). The strongest effect of inhibition using R5421 can be seen for A23187 activation. Inhibition of LPA- and PMA-activated PS exposure is less pronounced. The control value for RBCs showing PS exposure in the absence of any activating substance was 0.98 ± 0.15 % in the absence of R5421 and DMSO, 1.22 ± 0.18 % in the absence of R5421 and presence of DMSO, and 1.99 ± 0.80 % in the presence of R5421 and DMSO (n = 5, no pair of the 3 values shows a significant difference). Higher concentrations of R5421 (up to 1 mM) did not lead to significant higher reductions but caused strong haemolysis (data

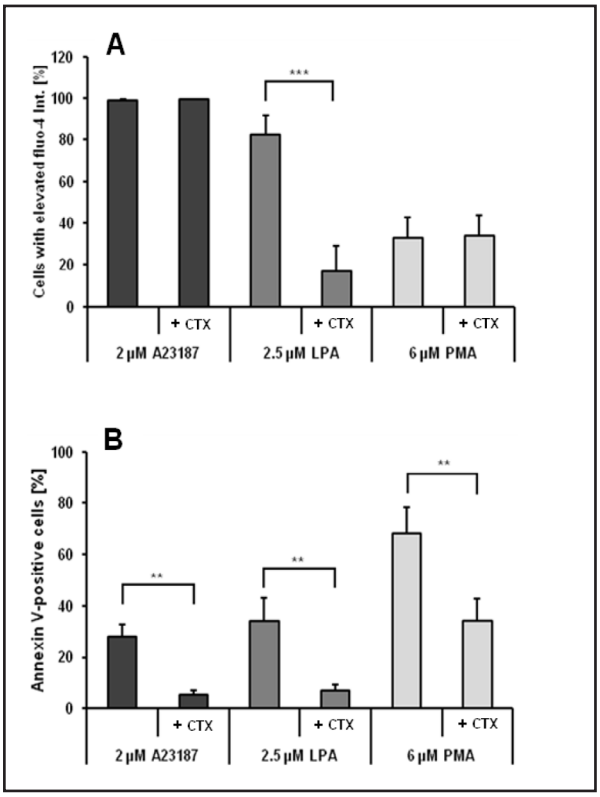

Fig. 4. Percentage of RBCs (A) responding with increased intracellular Ca^{2+} content (elevated fluo-4 intensity) and (B) responding with increased PS exposure (annexin V-positive cells) after activation with A23187 (2 µM) for 30 min, LPA (2.5 µM) for 1 min, or PMA (6 µM) for 30 min in the absence or presence of Gardos channel inhibitor charybdotoxin (CTX, 20 nM) using flow cytometry. Mean values of at least 3 different blood samples (3 x 30.000 cells), error bars = S.D. (only upper error bars are shown for convenience). Significant differences, ANOVA ($0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***)) are shown in the Figure. Please note that for the experiments presented in Figs. 1, 4, and 6 another LPA batch compared to experiments presented in Fig. 3 was used (this explains slight differences in the LPA control values, see also Material and Methods).

not shown). As can be seen from the images presented in Fig. 2, the inhibitor R5421 causes slight shape changes of the RBCs, most obvious after A23187 activation.

In case of A23187 activation and reduced $CaCl_2$ concentration, no significant increase of the amount of cells showing PS exposure could be observed at a Ca^{2+} concentration of 0.1 mM after 30 min compared to the control at time point zero (see above). For Ca^{2+} concentrations of 0.5 mM, 1 mM, and 2 mM the percentage of cells showing PS exposure was 9.89 ± 3.99 % (n = 3), 22.78 ± 4.06 % (n = 3), and 28.25 ± 4.02 % (n = 3), respectively. These values were obtained in a set of experiments different from data presented for A23187 activation in the presence of 2 mM Ca^{2+} in Figs. 1B, 3B, 4B, and 6.

Inhibition of the PKCα

Chelerythrine chloride and calphostin C have been used to inhibit the PKCα. Chelerythrine chloride is an inhibitor of the kinase domain of PKCα, whereas calphostin C blocks the PMA- and diacylglycerol (DAG)-binding site of the PKCα [41, 42, 44, 45].

The percentage of RBCs that show an increase in the intracellular Ca^{2+} content is not affected by an inhibition of the PKCα using chelerythrine chloride in case of A23187- and LPA-activation. In contrast, a significant reduction can be seen after PMA activation (Fig. 3A). The control value for RBCs with elevated Ca^{2+} content in the absence of any activating substance was 1.15 ± 0.30 % in the absence and 1.50 ± 0.72 % in presence of chelerythrine chloride (n = 3, not significantly different).

The situation for PS exposure after inhibition of the PKCα is slightly different. Although there is no significant change of the PS exposing RBCs after A23187 activation, a significant reduction of the cells showing PS exposure can be seen after activation with LPA or PMA (Fig. 3B). The control value for RBCs showing PS exposure in the absence of any activating substance was 1.13 ± 0.13 % in the absence and 1.24 ± 0.40 % in the presence of chelerythrine chloride (n = 3, not significantly different).

Using the PKCα inhibitor calphostin C, the following results have been obtained: The percentage of cells showing an increased Ca^{2+} content does not change significantly after A23187 activation but is slightly (but not significantly) decreased after LPA activation. The

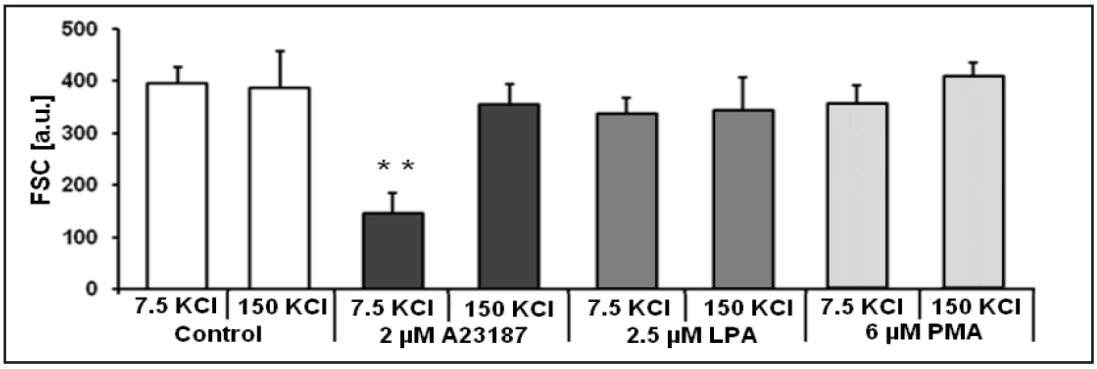

Fig. 5. Flow cytometry analysis (forward scatter, FSC) of RBCs after activation with A23187 (2 µM) for 30 min, LPA (2.5 µM) for 1 min, or PMA (6 µM) fo 30 min in normal physiological solution (HPS) containing 7.5 mM KCl, and solutions with higher KCl concentrations (compensated by a reduction of the NaCl concentration to keep the osmolarity constant) in comparison to control (absence of A23187, LPA, PMA). To all solutions 2 mM $CaCl_2$ were added. Mean values of at least 4 different blood samples (4 x 30.000 cells), error bars = S.D. (only upper error bars are shown for convenience). Significant differences, ANOVA ($0.001 < p \leq 0.01$ (**)) for the value measured after activation with A23187 in HPS containing 7.5 mM KCl *vs.* all other values.

decrease after PMA activation is much more pronounced and significant (Fig. 3A). The control value for RBCs with elevated Ca^{2+} content in the absence of any activating substance was 1.16 ± 0.16 % in the absence and 1.39 ± 0.68 % in presence of calphostin C (n = 3, not significantly different). The data for the PS exposure are comparable with the results obtained for the inhibition with chelerythrine chloride. No significant change of the PS exposing RBCs after A23187 activation has been observed but a significant reduction of the cells showing PS exposure can be seen after activation with LPA or PMA (Fig. 3B). The control value for RBCs showing PS exposure in the absence of any activating substance was 1.08 ± 0.03 % in the absence and 1.02 ± 0.32 % in the presence of calphostin C (n = 3, not significantly different).

We carried out experiments where RBCs have been activated with LPA and the PKCα has been inhibited by using chelerythrine chloride and calphostin C simultaneously. In this case, the percentage of cells showing an increased Ca^{2+} content was significantly reduced (Fig. 3A). The reduction of the PS exposing cells was even more pronounced compared with the situation of the inhibition using one of the two PKCα inhibitors alone (Fig. 3B). In addition, RBCs have been activated with PMA. The obtained data show that in this case a double inhibition using chelerythrine chloride and calphostin C does not lead to a larger inhibition of the percentage of RBCs with an elevated Ca^{2+} content compared with an inhibition using one of the substances alone (Fig. 3A). However, the percentage of RBCs with PS exposure is more pronounced compared with the data measured with one inhibitor only (Fig. 3B).

Simultaneous inhibition of the PKCα and the scramblase

In another set of experiments we inhibited the PKCα and the scramblase simultaneously using one PKCα inhibitor and the scramblase inhibitor R5421. In case of RBC activation with LPA, the simultaneous inhibition of the PKCα using chelerythrine chloride and the scramblase using R5421 did not result in a decrease of the percentage of the cells with a higher Ca^{2+} content but resulted in a dramatic decrease of the cells showing PS exposure (Fig. 3A, B). When the PKCα and the scramblase were inhibited simultaneously with calphostin C and R5421, respectively, a significant reduction of the percentage of cells with an enhanced intracellular Ca^{2+} content has been observed (Fig. 3A). For the PS exposure again a significant decrease can be seen like in cases of chelerythrine chloride and calphostin C or chelerythrine chloride and R5421 (Fig. 3B). The control values in the absence of the activating substances for all combinations of inhibition presented in Figs. 3A and 3B are not significantly different from the values obtained for inhibition with one of the substances.

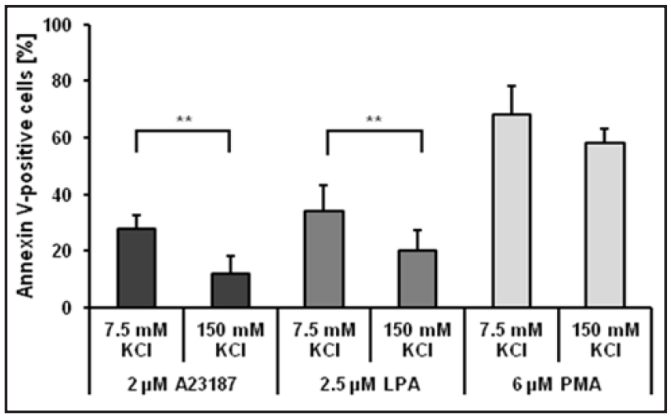

Fig. 6. Percentage of RBCs responding with increased PS exposure (annexin V-positive cells) after activation with A23187 (2 µM) for 30 min, LPA (2.5 µM) for 1 min, or PMA (6 µM) for 30 min in normal physiological solution (HPS) containing 7.5 mM KCl and a solution containing 150 mM KCl (compensated by a reduction of the NaCl concentration to keep the osmolarity constant, for detailed composition see Material and Methods) using flow cytometry. Mean values of at least 6 different blood samples (6 x 30.000 cells), error bars = S.D. (only upper error bars are shown for convenience). Significant differences, ANOVA ($0.001 < p \leq 0.01$ (**)) are shown in the Figure. Please note that for the experiments presented in Figs. 1, 4, and 6 another LPA batch compared to experiments presented in Fig. 3 was used (this explains slight differences in the LPA control values, see also Material and Methods).

Inhibition of the K^+ efflux via the Gardos channel and change of cell volume

To inhibit the Gardos channel, the classical inhibitor charybdotoxin has been applied [30, 41]. The inhibitor did not affect the percentage of cells showing an increased intracellular Ca^{2+} content after stimulation with A23187, i.e. nearly all RBCs showed an evaluated Ca^{2+} level in the absence or presence of charybdotoxin (Fig. 4A). The PS exposure, however, was reduced to a low level, i.e. close to values of the PS exposure obtained in the presence of the scramblase inhibitor R5421 (Fig. 1B *vs.* Fig. 4B). Interestingly, in case of LPA activation, the percentage of cells with an elevated intracellular Ca^{2+} content was significantly decreased and again PS exposure decreased to a very low level (Fig. 4A, B). In case of PMA activation, again the percentage of cells with increased Ca^{2+} content was not affected by charybdotoxin (Fig. 4A) but the PS exposure was significantly decreased in the presence of the inhibitor (Fig. 4B).

In another set of experiments, we increased the extracellular K^+ concentration of the HPS, compensating the osmolarity by reducing the Na^+ concentration. Elevation of the extracellular K^+ concentration reduces the efflux of K^+ through the Gardos channel, resulting in a decreased loss of KCl and osmotically obliged H_2O. This in turn leads to a diminished shrinkage of the cells. One can assume that an elevation of the extracellular KCl concentration to 150 mM (instead of 145 mM NaCl plus 7.5 mM KCl of the normal HPS the high K^+ HPS contains 150 mM KCl plus 2.5 mM NaCl) completely inactivates the K^+ efflux via the Gardos channel, i.e. the cell volume remains constant. The cell volume has been taken as the forward scatter (FSC) measured by flow cytometry. Data are presented in Fig. 5. One can see that the volume decrease is most pronounced in the 7.5 mM KCl solution in case of A23187 activation. The percentage of RBCs showing an increased intracellular Ca^{2+} content does not change in solution of high extracellular K^+ content (high K^+ HPS) in all 3 cases of activation (LPA, PMA, A23187) compared to the normal HPS (data not shown). However, as shown in Fig. 6, an inhibition of the K^+ efflux in high K^+ HPS is able to significantly reduce the A23187- as well as the LPA-induced PS exposure. In contrast, the PMA-induced PS exposure is not affected by high K^+ HPS (Fig. 6). In a separate set of experiments the addition of charybdotoxin to the normal and high K^+ HPS led to a significant reduction of the percentage of RBCs showing PS exposure after PMA activation from 63.79 ± 5.31 % to 33.18 ± 8.87 % (n = 3) and from 57.55 ± 4.95 % to 32.27 ± 7.88 % (n = 3), respectively. The corresponding values in the absence and presence of charybdotoxin are not significantly different.

To investigate a possible effect of the cell volume on the intracellular Ca^{2+} content as well as PS exposure of RBCs, the cells were shrunken by adding 30 mM sucrose to the HPS and swollen by using the HPS with reduced NaCl concentration (130 mM NaCl instead of 145 mM). Such a procedure was introduced by Dunham and Ellory [46] and used thereafter

e.g. to increase the RBC volume for stimulation of the K,Cl cotransport (e.g., [47]). There is neither a significant change in the percentage of RBCs with elevated intracellular Ca^{2+} content nor with PS exposure compared to control (data not shown). However, the change of the cell volume after such treatment is much smaller compared to the situation of Gardos channel activation using A23187 (see above and Fig. 6). Indeed, FSC measurements of RBCs in the HPS solution containing additionally 30 mM sucrose showed only a 6.1 ± 2.2 % (n = 3) reduction of this parameter compared to RBCs in normal HPS, whereas the reduction was 60.2 ± 11.4 % (n = 3) in normal HPS after stimulation with A23187 (Fig. 5). In HPS with reduced NaCl content (130 mM) the FSC increased by 7.7 ± 3.2 % (n = 3) compared to control.

Discussion

Based on the previous knowledge about PS exposure in RBC (see Introduction) we could gain further insight into this multimodal process making use of the stimulating agents A23187, LPA and PMA, the inhibitors R5421 (scramblase), chelerythrine chloride and calphostin C (both for PKCα), charybdotoxin (Gardos channel) as well as by modulation of the ion content of the extracellular solutions.

To start the discussion, we first like to consider the experiments where initially cells were exclusively challenged with a Ca^{2+} increase by adding the Ca^{2+} ionophore A23187 in the presence of 2 mM extracellular Ca^{2+}. Under all conditions measured (independent of any inhibitor) the entire RBC population showed an intracellular Ca^{2+} increase (Figs. 1A, 3A, 4A). Such a relative high Ca^{2+} concentration served as positive control and has been used in previous studies. Nevertheless, the stimulation result of nearly all RBCs showing increased intracellular Ca^{2+} content was maintained for reduced Ca^{2+} concentrations (down to 0.1 mM) and reduced A23187 incubation times (down to 1 min). The response of only approximately one third of the cells presenting detectable PS in the outer membrane leaflet at 2 mM extracellular Ca^{2+} confirms previous studies [3, 25, 38]. However, a reduction of the extracellular Ca^{2+} concentration results in a significant decrease of the amount of RBCs showing PS exposure. RBC age does not influence PS exposure after short-time incubation [37]. For a putative explanation please refer to the discussion of the Gardos channel related measurements outlined below. Scramblase inhibitor R5421 could suppress PS exposure from one third to about 8 % of the RBCs (Fig. 1B). Whether the remaining 8 % are due to an incomplete inhibition of the scramblase or caused by a different Ca^{2+}-dependent mechanism remains unclear. As already mentioned in the Results section, a higher concentration of R5421 does not lead to a further scramblase inhibition but to a significant increase in haemolysis.

PKCα inhibitors do not significantly change the A23187 induced PS exposure (Fig. 3B). This let us conclude that although intracellular Ca^{2+} entry is mediating PKCα translocation to the plasma membrane - a requirement for PKCα activation [48], the Ca^{2+} increase alone is not sufficient to activate significant amounts of PKCα. Additional membrane binding of PKCα's C1 domain is necessary for activation [34]. Alternatively, Ca^{2+} at these levels does not require PKCα activity for their action on scrambling.

Interestingly, charybdotoxin inhibits the Ca^{2+}-induced PS exposure to approximately the same level as the R5421 does (Fig. 4B), indicating that Gardos channel activity is required for the scramblase activity. As it is hard to imagine that a rather limited drop in the intracellular K^+ concentration causes an inhibition of the scramblase, a more severe effect of Gardos channel activation is the hyperpolarization of the cell [49]. The low abundance of the Gardos channel and its heterogeneous distribution among the cells [50, 51] may induce a hyperpolarization only in a subpopulation of the cells and such provide an explanation why only one third of the high Ca^{2+} cells respond with a PS exposure. The experimental conditions where RBCs were challenged with A23187 in the presence of 150 mM KCl in the extracellular solution would activate the Gardos channel without a hyperpolarization (due

to the changed K^+ gradient, K^+ loss is prohibited). The decreased PS exposure in A23187 stimulated RBCs in 150 mM KCl solution (Fig. 6) supports the hypothesis that activation of the scramblase requires a hyperpolarization. A further explanation involving the Gardos channel could be based on the induced cell shrinkage (Fig. 5) and associated membrane tension. However, much less pronounced cell shrinkage during stimulation with LPA compared to A23187 treatment (Fig. 2), which depict the same Gardos channel dependence, render this explanation unlikely. Alternatively, one has to take into consideration that a high extracellular K^+ content itself can lead to a reduction of the PS exposure in RBCs as has been demonstrated [31, 52]. In addition, shrinkage of RBCs may act via ceramide [53].

The stimulation of RBCs with LPA is a multimodal effect involving different signalling cascades already for the Ca^{2+} entry [25, 54], which will be subject of further investigations.

As discussed above, the Ca^{2+}-dependent PS exposure can be attributed to the scramblase activity. Comparing PS exposure after LPA simulation for R5421 modulation, charybdotoxin application and 150 mM external KCl (Figs. 1B, 4B, 6) support the dependence of the scramblase on the Gardos channel induced hyperpolarization as proposed above.

In similarity, also a significant part of the LPA-induced Ca^{2+} entry depends on the Gardos channel (Fig. 4A). Since the LPA-induced Ca^{2+} entry is believed to be at least partly mediated by the $Ca_V2.1$ channel [25, 55] the dependence on the Gardos channel is sensible: Similar as hypothesized for the scramblase, it would enable the hyperpolarization required for the opening procedure of the $Ca_V2.1$ channel [56]. At the resting membrane potential of RBCs (approximately -10 mV), $Ca_V2.1$ is inactivated. To enable an opening of the channel a hyperpolarization is necessary to transfer the channel from the inactivated into a closed state. Therefore the Gardos-channel activation and the associated hyperpolarization could set the prerequisite for $Ca_V2.1$ to open. Openings of further, e.g. non-selective cation channels could then result in a further depolarization of the RBCs that could possibly open $Ca_V2.1$. This charybdotoxin dependent Ca^{2+} increase directly translates in a decreased PS exposure upon LPA stimulation in the presence of charybdotoxin (Fig. 4).

A second mechanism of LPA induced PS exposure, less or indirect dependent of Ca^{2+}, is PKCα related [3, 25]. This perspective can be supported comparing the PS exposure after LPA stimulation in the presence of the scramblase inhibitor R5421 (Fig. 1B) with the LPA stimulation in the presence of the PKCα inhibitors (Fig. 3B). However, scramblase and PKCα inhibition is not strongly additive (Fig. 3B), suggesting some degree of interaction in the PS exposing processes.

Furthermore, the reason for R5421 modulation of LPA- and PMA-induced Ca^{2+} entry (Fig. 1A) remains elusive. However, one should take into consideration that inhibitors have multiple actions, i.e. present unspecific effects.

In contrast, it is well known that PMA itself has the potential to induce Ca^{2+} signals [57] and as can be seen from Fig. 3A, part of this Ca^{2+} entry is PKCα-mediated, while a 2nd portion is due to unspecified effects of PMA. As a result of this, also the PMA-induced PS exposure involves a Ca^{2+}-dependent scramblase-mediated portion and a direct PKCα-induced PS translocation. This interpretation is compatible with the inhibition experiments of the scramblase using R5421 (Fig. 1B) and the PKCα using chelerythrine chloride and calphostin C (Fig. 3B). Nevertheless we see differences in PS exposure (i) if only one domain of the PKCα is blocked (kinase domain by chelerythrine chloride or PMA and DAG binding C1 domain by calphostin C) or (ii) if both domains are inhibited simultaneously (Fig. 3B). This difference is independent whether cells were stimulated with LPA or PMA. Since a block of the C1 domain should prevent PKCα binding to the membrane and inhibition of the kinase domain should prevent phosphorylation, both inhibitors should prevent the phosphorylation of membrane proteins. Therefore, the above mentioned differences can only be explained by an incomplete inhibition. The involvement of phosphorylation of cytosolic proteins can be excluded since the membrane binding is required for the activation of PKCα [34]. Throughout all conditions, the PS exposure caused by PMA was always more pronounced than the one initiated by LPA (Figs. 1B, 3B, 4B and 6) because PMA leads to a direct (artificial) activation of PKCα, while LPA triggers a signalling cascade with an eventual activation of PKCα [54].

Conclusions

PS exposure is mediated by the Ca^{2+}-dependent scramblase but also by PKCα activated by LPA and PMA in a Ca^{2+}-dependent and a Ca^{2+}-independent manner. We hypothesize that a hyperpolarisation of RBCs caused by the opening of the Gardos channel is essential for the scramblase activity as well as for a fraction of the LPA-induced Ca^{2+} entry. The signalling cascades leading to LPA-induced Ca^{2+} entry need further investigations.

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Disclosure Statement

The authors declare no conflict of interest.

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